DEPARTMENT OF THE AIR FORCE
AIR FORCE RESEARCH LABORATORY
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CO-LAB REPORT

15 June 2000

MEMORANDUM FOR US EPA
NCEA (MD-52)
RTP, NC 27711
ATTN: ANNIE M. JARABEK

FROM: AFRL/HEST
Operational Toxicology Branch
2856 G St. Bldg 79
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SUBJECT: Consultative Letter, AFRL-HE-WP-CL-2000-0034, Thyroid Hormone and TSH Co-Laboratory Study Report

1. A study was conducted to compare serum thyroid hormone and TSH data obtained by radioimmunoassay (RIA) procedures for three different research laboratories that participated in perchlorate toxicity studies involving hormone analysis. The report, "Thyroid Hormone and TSH Co-Laboratory Study Report," is an attachment to this consultative letter. In addition, individual values for T_3, T_4 and TSH for each laboratory are attached as Appendices A, B and C corresponding to each participating laboratory.

2. Reproducibility across laboratories was examined statistically by determining reproducibility limits for each sample and for each hormone. The reproducibility varied for each hormone with T_3 showing the best reproducibility and TSH the least. For T_3, laboratories A and B were in closer agreement overall than laboratory C. Laboratories B and C were closer for four out of six T_4 samples while laboratories A and B were closer for the other two samples. For TSH, laboratories A and B were closer for five out of six samples. In general, laboratory B had the closest repeatability for all three hormones. Laboratory C varied in repeatability for T_3 and T_4 but was very consistent for TSH. However, for TSH the overall mean for C varied from the other two laboratories. Although Laboratory A agreed closely to laboratory B in reproducibility, repeatability varied more for A than laboratories B and C for all three hormones. The difference between laboratory A as compared to laboratories B and C is the use of three replicates for hormone analysis by laboratory A. Three replicates ensure a more reproducible sample value even when repeatability is not as consistent.

3. For further information, I can be reached at 937-255-5150, extension 3163.

Latha Narayanan
Operational Toxicology Branch
Human Effectiveness Directorate

Attachments
1. Thyroid Hormone and TSH Co-Laboratory Study Report
2. Appendices A, B, C

1st Ind, AFRL/HEST 15 June 2000

MEMORANDUM FOR US EPA
ATTN: MS. ANNIE JARABEK

This letter report has been coordinated at the branch level and is approved for release.

David R. Mattie

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Thyroid Hormone and TSH Co-Laboratory Study Report

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INTRODUCTION

The production and storage of ammonium perchlorate (AP) has resulted in contamination of soil, ground and drinking water in a number of states (Urbansky, 1998). The perchlorate ion interferes with iodide uptake in the thyroid resulting in reduced thyroidal hormone synthesis. This leads to lowered T_3 and T_4 blood levels and TSH activation to stimulate production of thyroid hormones (Wolff, 1998). In order to study perchlorate induced TSH and thyroid hormonal changes, several research laboratories performed the analysis of TSH and thyroid hormones in rodent serum using radioimmunoassay (RIA) procedures. The Environmental Protection Agency (EPA) and external peer reviewers expressed their concerns regarding the variability present in the rodent TSH and thyroid hormone data obtained from the research laboratories involved. In order to evaluate the potential toxicity of perchlorate accurately, a good correlation for rodent TSH and thyroid hormone analyses from different perchlorate research groups involved is essential. Perchlorate project scientists realized the need for a collaborative study for TSH and thyroid hormone analyses in rodent serum in order to account for the interlaboratory data variability and to correlate laboratory results. The purpose of this study was to correlate serum thyroid hormone and TSH data obtained by radioimmunoassay (RIA) procedures for three different research laboratories.

Hormone Analysis

The following serum hormone levels were analyzed: total thyroxine (T_4), triiodothyronine (T_3) and thyroid stimulating hormone (TSH). RIA assay kits from the same batch number and with the same expiration date were used for all thyroid hormone or TSH measurements for all the standard and unknown samples. For unknown samples, six rat serum samples, plus six samples obtained from different species were used. Sources of the RIA kits were:

1) Three canine T_3 RIA assay kits were purchased from Diagnostic Product Corp. (Los Angeles, CA) and were sent to three laboratories designated as A, B and C (one kit per lab).
2) Three rat T_4 RIA assay kits were purchased from Diagnostic Product Corp. (Los Angeles, CA) and were sent to laboratories A, B and C (one kit per lab).
3) Three rat TSH RIA assay kits were purchased from Amersham Corp. (Arlington Heights, IL) and were sent to laboratories A, B and C (one kit per lab).

The unknown samples were purchased as serum from Sigma (St Louis, MO).

- Six unknown serum samples (rat, rat diluted 25%, dog, guinea pig, rabbit and mouse) were sent to laboratories A, B and C for analysis of T_3 and T_4.
- Six unknown rat serum samples were sent to laboratories A, B and C for analysis of TSH. The first sample was spiked with a TSH standard and five dilutions were then made to obtain the other five samples. The dilutions were 0.08, 0.16, 0.32, 0.64 and 1.28, respectively.

Assays for T_3, T_4 and TSH were performed using the RIA kits according to manufacturers' and each laboratories' standard procedures. Two labs divided the sample into three parts or replicates (lab C used two parts) for the analysis. The replicate CPM (counts per minute)

values were averaged to determine the T_3, T_4, and TSH levels of each sample. All sample data for T_3, T_4 and TSH analyses from the three laboratories were submitted to AFRL/HEST, who served as the sponsor.

Statistics

The purpose of the analysis was to investigate the reproducibility (i.e., variability across labs) of the hormones and repeatability (i.e., variability within labs) of the counts per minute (CPM). The first section of this report addresses reproducibility while the second part looks at repeatability.

RESULTS AND DISCUSSION

Reproducibility

Table 1 contains the hormone levels for each sample and lab. The purpose of the analysis was to determine how similar the means were across the labs. To do this, the reproducibility limit was determined for each hormone and sample. Reproducibility limit (RL) is defined as follows: approximately 95% of all pairs of means from the same hormone, same sample and different labs should differ in absolute value by less than the RL. The difference in means between any two labs is a normally distributed random variable with mean 0. The range ± RL is then the middle 95% for this distribution (i.e., 2.5% in each tail). Table 2 contains the reproducibility limits for each hormone and sample.

TABLE 1. HORMONE ANALYSIS RESULTS FOR THE THREE LABORATORIES

	T_3 (ng/dL)			T_4 (μg/dL)			TSH (ng/mL)		
Sample	Lab A	Lab B	Lab C	Lab A	Lab B	Lab C	Lab A	Lab B	Lab C
1	46.08	47.39	59.60	1.84	1.31	1.35	5.84	5.46	4.93
2	42.75	42.14	51.10	1.89	1.55	1.50	6.53	5.74	4.29
3	170.71	170.51	178.00	3.33	3.43	3.45	8.44	8.28	6.35
4	118.01	118.21	113.00	1.78	1.89	1.96	11.17	10.97	8.23
5	93.12	95.13	93.26	2.83	2.98	2.71	13.59	12.87	15.60
6	134.69	139.36	135.60	3.21	3.37	2.90	19.38	15.98	27.05

TABLE 2. REPRODUCIBILITY LIMITS (RL) FOR EACH HORMONE AND SAMPLE

Hormone	Sample	Mean	RL	RL % of Mean
T_3 (ng/dL)	1	51.02	20.65	40
	2	45.33	13.87	31
	3	173.07	11.82	7
	4	116.41	8.18	7
	5	93.82	3.15	3
	6	136.55	6.86	5
T_4 (μg/dL)	1	1.50	0.82	55
	2	1.65	0.59	36
	3	3.40	0.18	5
	4	1.88	0.25	13
	5	2.84	0.37	13
	6	3.16	0.66	21
TSH (ng/mL)	1	5.41	1.27	23
	2	5.52	3.15	57
	3	7.69	3.22	42
	4	10.12	4.55	45
	5	14.02	3.92	28
	6	20.80	15.71	76

The RL theory for each hormone and sample is as follows:

`1 2

Model: Let $Y_i = \mu + L_i + \varepsilon_i$ i=1,2,3 where L=Lab

The reproducibility limit (RL) is such that: Prob{$|Y_i - Y_{i'}| \leq RL$} = 0.95 $i \neq i'$

The variance of $Y_i - Y_{i'} = \sqrt{2(\sigma_L^2 + \sigma_\epsilon^2)}$. Substituting the estimate of $\sigma_L^2 + \sigma_\varepsilon^2 = S_L^2$ = the sample variance of the means from the 3 labs, then: $RL = 1.96\sqrt{2S_L^2} = 2.77\sqrt{S_L^2}$.

Note that if there were other factors besides lab, or if there were replications, the variance estimate would not equal 2*(sample variance), it would be 2*(a function of mean squares).

There are no steadfast rules for what constitutes a reasonable RL (expressed as a % of the mean). Experience with measuring human visual systems has shown RL % of mean to be 7-15%. Results shown in Table 2 are mixed. Samples 3 through 6 for T_3 and sample 3 for T_4 (maybe samples 4 and 5 also) indicate close agreement among the laboratories. The remaining samples for T_3 and T_4 and all the samples for TSH do not show close agreement among the laboratories. As shown in Figures 1b, 2b and 3b, the absolute differences (expressed as % of mean) are all much less than the percents shown in Table 2.

However, with only three laboratories, the RL will be inflated. This is due to (n-1) used in the denominator of the unbiased variance estimate. As the number of labs increases, (n-1) approaches n so that the RL is not inflated. Since this study was trying to compare only three laboratories, inflated RLs could not be avoided.

Figures 1, 2 and 3 contain data from Table 1 along with the absolute differences between each pair of labs expressed as a percent of the mean. The mean is the average of the three labs.

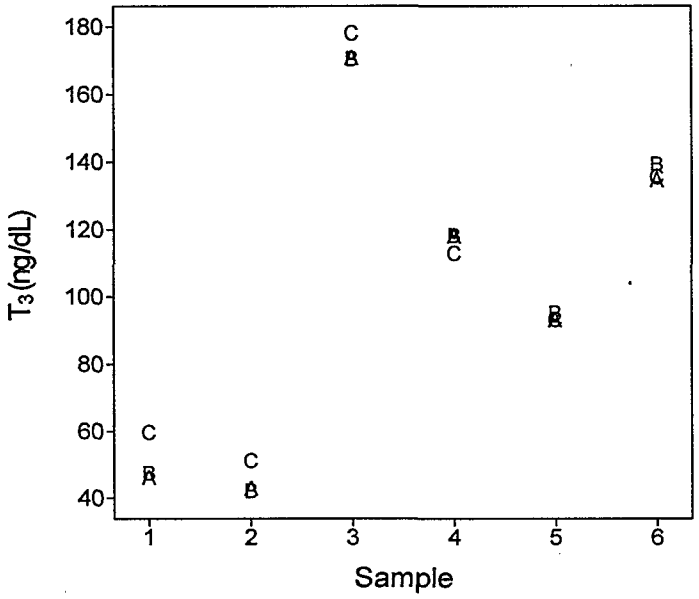

Figure 1a. T_3 for each sample and lab. Legend is value of lab.

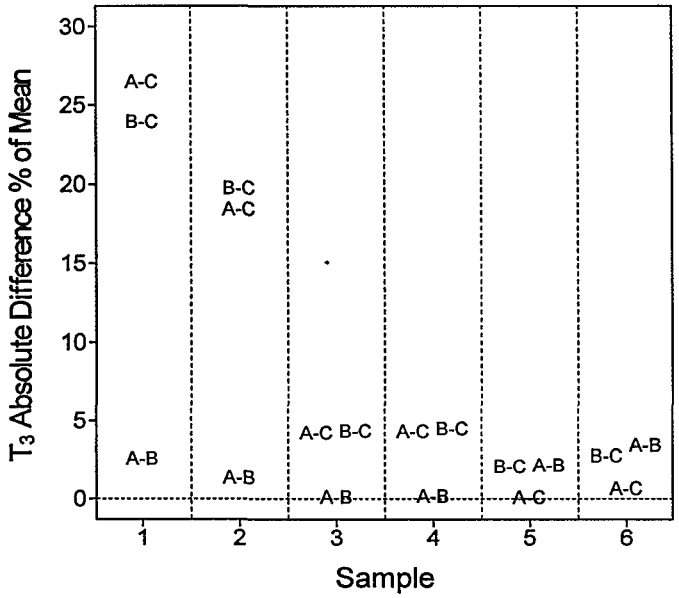

Figure 1b. Absolute difference between labs for T_3 as a percent of mean

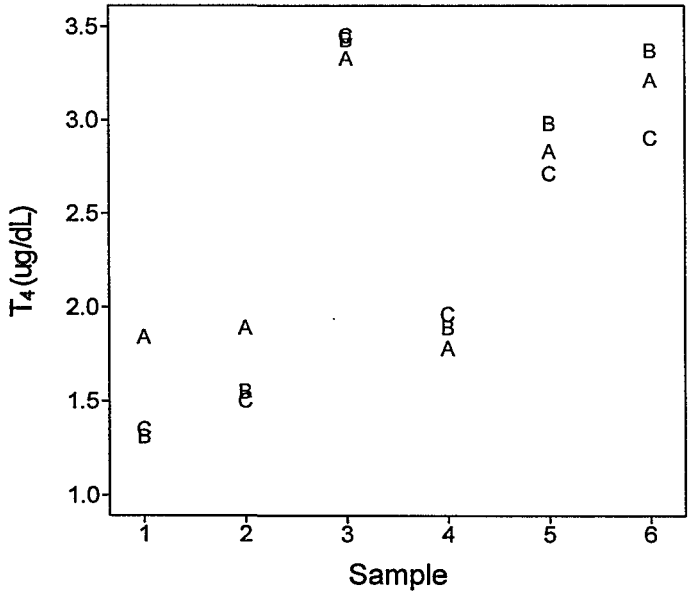

Figure 2a. T_4 for each sample and lab. Legend is value of lab.

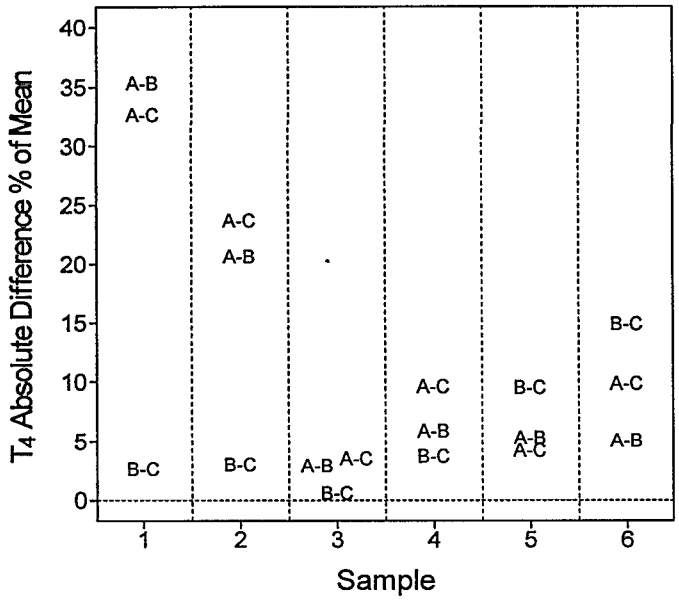

Figure 2b. Absolute difference between labs for T_4 as a percent of mean

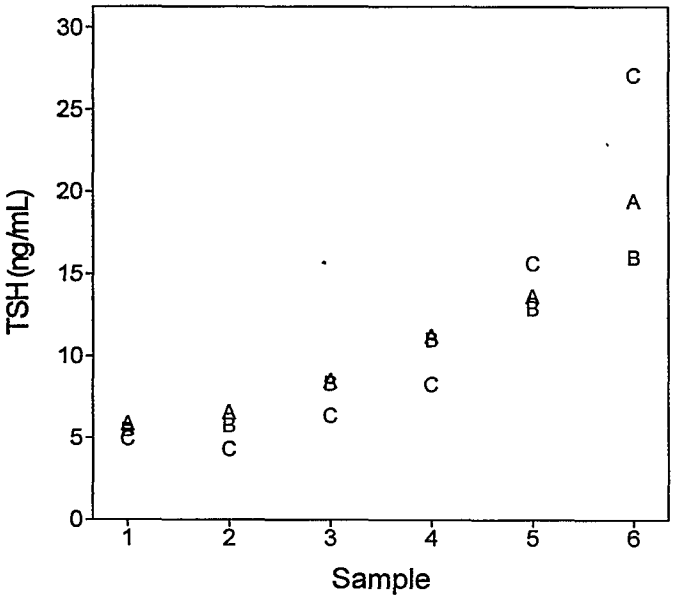

Figure 3a. TSH for each sample and lab. Legend is value of lab.

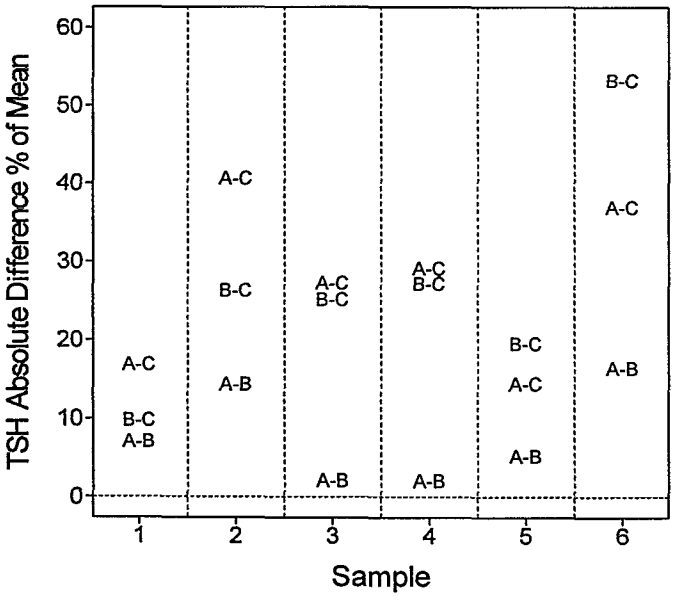

Figure 3b. Absolute difference between labs for TSH as a percent of mean

Repeatability

Table 3 (and Figures 4, 5 and 6) contains the CPM for each hormone, sample and lab. Figures 4b, 5b, and 6b show absolute differences between the CPM for each hormone, sample and lab expressed as a percent of the mean (i.e., average of the two or three replicates).

TABLE 3. COUNTS PER MINUTE (CPM) FOR EACH HORMONE, SAMPLE AND LAB. SOME VALUES WERE ROUNDED.

Hormone	Sample	Lab A			Lab B		Lab C	
		CPM1	CPM2	CPM3	CPM1	CPM2	CPM1	CPM2
T_3	1	4806	4813	4226	4281	4166	4111	4397
	2	3984	5270	4793	4374	4258	4154	4667
	3	3576	3729	3014	2972	2883	3175	3102
	4	4042	3799	3472	3393	3289	3553	3650
	5	4265	3359	4327	3597	3548	3929	3665
	6	3517	3649	3791	3232	3086	3316	3515
T_4	1	14332	15200	13531	14554	14239	15323	15257
	2	15031	13584	14140	13958	14158	15526	14334
	3	12023	11927	12965	11801	12061	12606	11848
	4	15808	13138	14426	13411	13833	14019	14118
	5	12902	12871	12818	12442	12303	13317	12724
	6	13188	12620	11497	11845	12141	12482	13113
TSH	1	5650	5536	5620	5883	4166	2838	2742
	2	5445	5302	5576	5751	5587	3035	2740
	3	4987	5057	5156	4999	5100	2636	2597
	4	4625	4790	4560	4647	4450	2466	2410
	5	4542	4207	4372	4559	3990	2079	1915
	6	4352	3523	3694	3999	3723	1567	1669

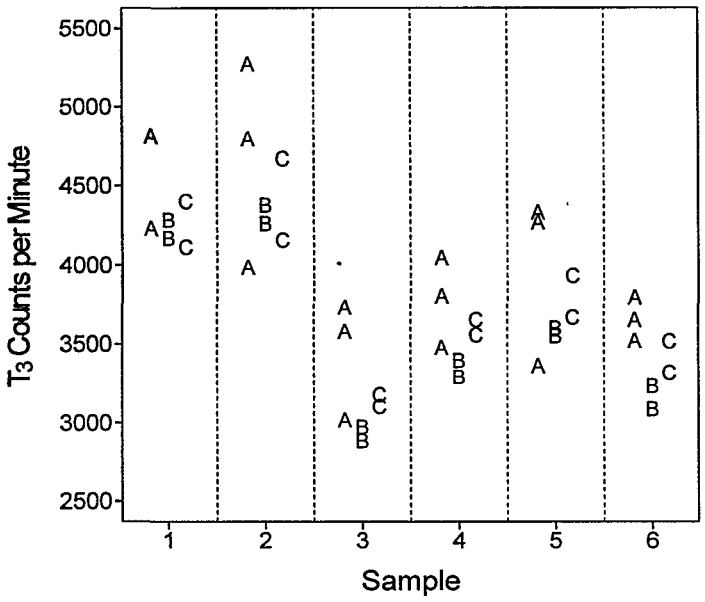

Figure 4a. T_3 counts per minute for each sample and lab. Legend is value of lab.

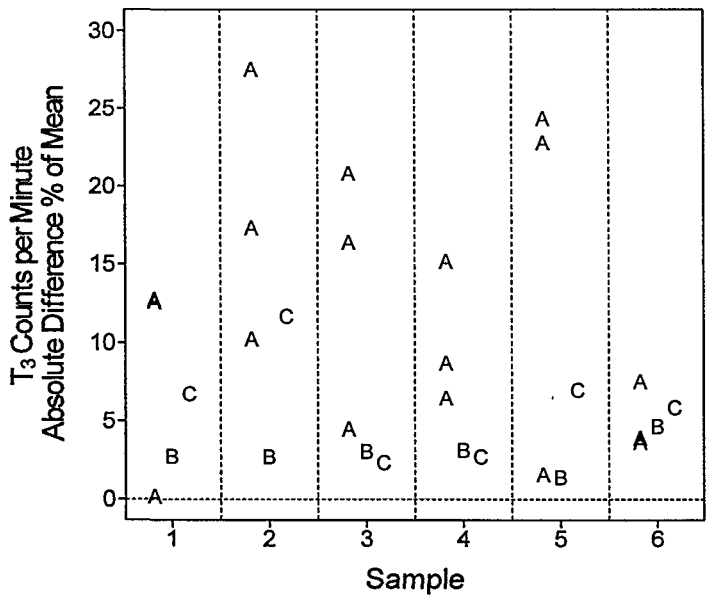

Figure 4b. Absolute difference between parts for T_3 (counts per minute) as a percent of mean. Legend is value of lab.

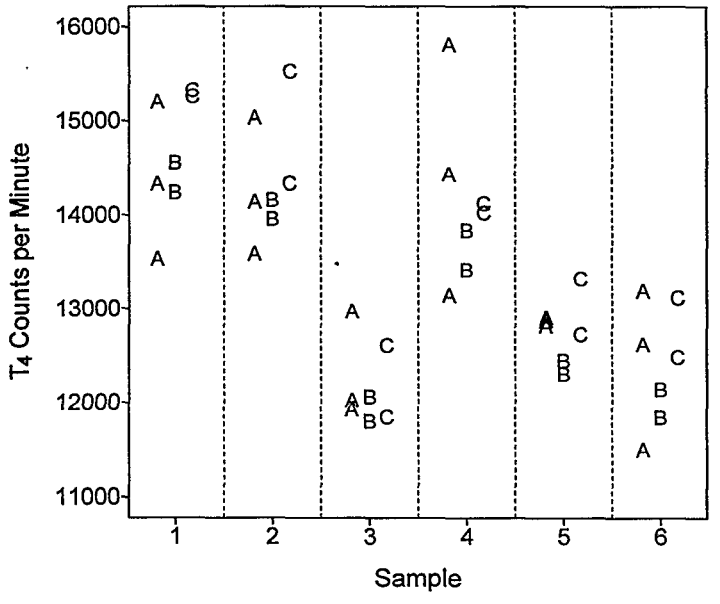

Figure 5a. T_3 counts per minute for each sample and lab. Legend is value of lab.

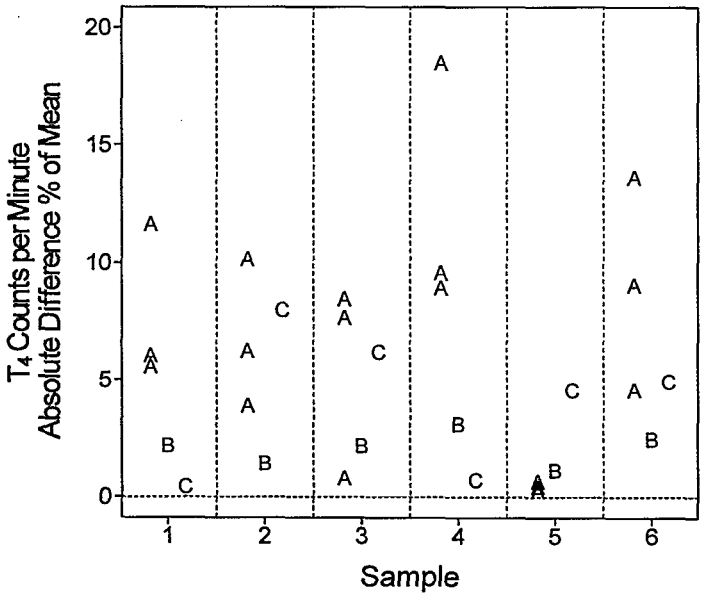

Figure 5b. Absolute difference between parts for T_4 (counts per minute) as a percent of mean. Legend is value of lab.

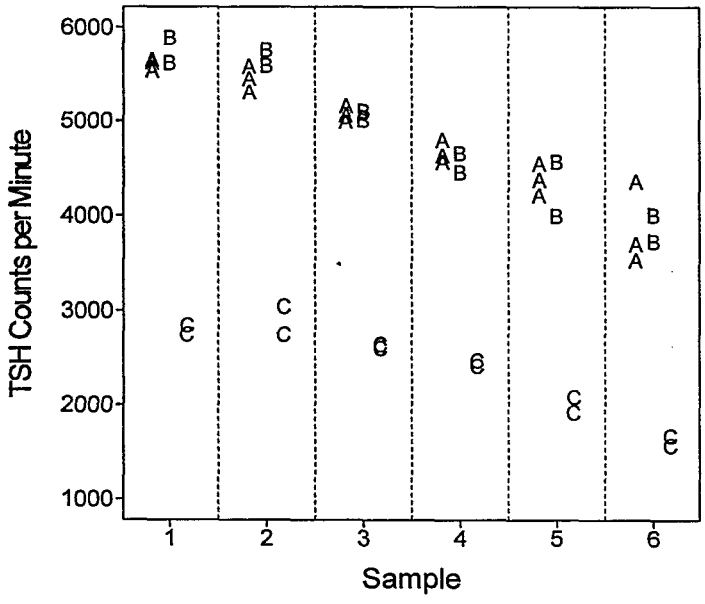

Figure 6a. TSH counts per minute for each sample and lab. Legend is value of lab.

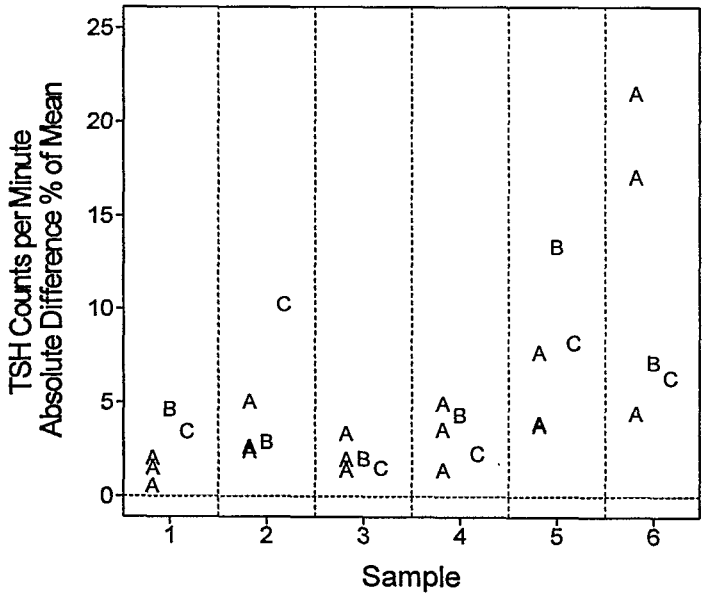

Figure 6b. Absolute difference between parts for TSH (counts per minute) as a percent of mean. Legend is value of lab.

SUMMARY AND CONCLUSIONS

Reproducibility across laboratories was examined statistically by determining reproducibility limits for each sample and for each hormone. The reproducibility varied for each hormone with T_3 showing the best reproducibility and TSH the least. For T_3, laboratories A and B were in closer agreement overall than laboratory C. Laboratories B and C were closer for four out of six T_4 samples while laboratories A and B were closer for the other two samples. For TSH, laboratories A and B were closer for five out of six samples. In general, laboratory B had the closest repeatability for all three hormones. Laboratory C varied in repeatability for T_3 and T_4 but was very consistent for TSH. However, for TSH the overall mean for C varied from the other two laboratories. Although Laboratory A agreed closely to laboratory B in reproducibility, repeatability varied more for A than laboratories B and C for all three hormones. The difference between laboratory A as compared to laboratories B and C is the use of three replicates for hormone analysis by laboratory A. Three replicates ensure a more reproducible sample value even when repeatability is not as consistent.

REFERENCES

Urbansky, E.T., 1998, Perchlorate chemistry: implications for analysis and remediation: Bioremed.J., 2, p. 81-95.

Wolff, J., 1998, Perchlorate and the Thyroid Gland: Pharmacolog.Rev., 50, p. 89-105.

APPENDIX A. T_4, T_3 AND TSH RESULTS FROM LABORATORY A

Unknown serum samples T_4 lab A	CPM1	CPM2	CPM3	Mean CPM-BKG	% Bound	T_4 conc in µg/dL
Sample #1	14332	15200	13531	13581.00	71.17	1.84
Sample #2	15031	13584	14140	13478.34	70.63	1.89
Sample #3	12023	11927	12965	11531.67	60.43	3.33
Sample #4	15808	13138	14426	13684.00	71.71	1.78
Sample #5	12902	12871	12818	12090.34	63.36	2.83
Sample #6	13188	12620	11497	11661.67	61.11	3.21

Unknown serum samples T_3 lab A	CPM1	CPM2	CPM3	Mean CPM-BKG	% Bound	T_3 conc in ng/dL
Sample #1	4806	4813	4226	4292.50	74.30	46.08
Sample #2	3984	5270	4793	4359.83	75.47	42.75
Sample #3	3576	3729	3014	3117.17	53.96	170.71
Sample #4	4042	3799	3472	3448.50	59.69	118.01
Sample #5	4265	3359	4327	3661.17	63.37	93.12
Sample #6	3517	3649	3791	3329.83	57.64	134.69

Unknown serum samples TSH lab A	CPM1	CPM2	CPM3	BKG	Mean CPM-BKG	% Bound	TSH conc in ng/100 µL	TSH conc in ng/mL
Sample #1	5650	5536	5620	1027	4575.00	60.22	0.58	5.84
Sample #2	5445	5302	5576	1027	4414.00	58.10	0.65	6.53
Sample #3	4987	5056.9		1027	2320.97	30.55	2.75	27.49
Sample #4	4625	4790	4560	1027	3631.33	47.79	1.12	11.17
Sample #5	4542	4207	4372	1027	3346.67	44.05	1.36	13.59
Sample #6	4352	3523	3694	1027	2829.33	37.24	1.94	19.38

APPENDIX B. T_4, T_3 AND TSH RESULTS FROM LABORATORY B

lab B

		replicates			
T_4					
	µg/dL	cpm	µg/dL	cpm	µg/dL
1	1.31	14554	1.20	14239	1.42
2	1.55	13958	1.63	14158	1.48
3	3.43	11801	3.57	12061	3.30
4	1.89	13411	2.05	13833	1.72
5	2.98	12442	2.91	12303	3.05
6	3.37	11845	3.52	12141	3.21
T_3	ng/dL	cpm	ng/dL	cpm	ng/dL
1	47.39	4281	44.01	4166	50.77
2	42.14	4374	38.94	4258	45.35
3	170.51	2972	163.95	2883	177.08
4	118.21	3393	112.60	3289	123.83
5	95.13	3597	92.90	3548	97.37
6	139.36	3232	130.34	3086	148.38
TSH	ng/mL	cpm	ng/mL	cpm	ng/mL
1	5.46	5883	5.00	5617	5.92
2	5.74	5751	5.44	5587	6.03
3	8.28	4999	8.52	5100	8.04
4	10.97	4647	10.37	4450	11.56
5	12.87	4559	10.88	3990	14.85
6	15.98	3999	14.77	3723	17.18

APPENDIX C. T_4, T_3 AND TSH RESULTS FROM LABORATORY C

lab C

T_3

Sample ID	CPM Rep1	CPM Rep 2	CPM Average	ng/dL Rep1	ng/dL Rep 2	ng/dL Average (ng/dL)*
1	4111	4397	4254	68.52702	51.75421	59.55
2	4154	4667	4410.5	65.69492	39.70543	51.07
3	3175	3102	3138.5	171.7356	184.4925	178.00
4	3553	3650	3601.5	118.5022	107.74	112.99
5	3929	3665	3797	81.93036	106.1654	93.26
6	3316	3515	3415.5	149.5391	123.0056	135.63

*ng/dL of T_3 calculated from the average of the CPM reps

T_4

Sample ID	CPM Rep1	CPM Rep 2	CPM Average	µg/dL Rep1	µg/dL Rep 2	Average (µg/dL)*
1	15323	15257	15290	1.33214	1.359471	1.35
2	15526	14334	14930	1.251473	1.805998	1.50
3	12606	11848	12227	3.073566	3.880964	3.45
4	14019	14118	14068.5	1.989816	1.930114	1.96
5	13317	12724	13020.5	2.469599	2.963966	2.71
6	12482	13113	12797.5	3.193107	2.629592	2.90

*µg/dL of T_4 calculated from the average of the CPM reps

TSH

Sample ID	CPM Rep1	CPM Rep 2	CPM Average	ng/mL Rep1	ng/mL Rep 2	Average (ng/mL)*
1	2838.3	2741.9	2790.1	4.601085	5.292057	4.93
2	3035	2739.6	2887.3	3.458385	5.309753	4.29
3	2636.1	2596.6	2616.35	6.170409	6.534498	6.35
4	2465.8	2410.3	2438.05	7.900599	8.563348	8.23
5	2078.6	1915.3	1996.95	13.8588	17.56546	15.60
6	1566.7	1668.9	1617.8	29.1336	25.11736	27.05

APPENDIX C. T_4, T_3 AND TSH RESULTS FROM LABORATORY C

*ng/mL of TSH calculated from the average of the CPM reps